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Chapter 4

*Angiogenic activation of
endothelial cells impairs efficacy of
VEGFR2 inhibitor treatment
in vitro*

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ABSTRACT

Purpose

In the complex environment of a tumor, endothelial cells are present in different stages of angiogenic activation, which may affect their responsiveness to antiangiogenic therapy. We investigated the consequences of angiogenically activating endothelial cells with VEGF prior to VEGFR2 inhibitor exposure *in vitro* for efficacy of drug treatment. In this way, we mimicked the *in vivo* situation of tumor endothelial cells that are exposed to VEGF before VEGFR2 inhibitor therapy is initiated.

Experimental design

HUVEC were treated with the VEGFR2 tyrosine kinase inhibitors vandetanib (ZD6474), vatalanib (PTK787/ZK222584) and SU6668, prior to or after stimulation with VEGF. Their responsiveness to VEGFR2 inhibition was determined by analyzing mRNA expression of the immediate-early VEGF-responsive genes EGR3, NR4A1, NR4A3 and COX-2. Furthermore, we investigated the underlying kinetics of phosphorylation of VEGFR2 and its downstream kinases ERK, p38 MAPK, and Akt in response to VEGFR2 inhibitor treatment prior to or after VEGF exposure.

Results and conclusions

We demonstrated that vandetanib and vatalanib inhibited expression of EGR3, NR4A1, NR4A3 and COX-2, in contrast to SU6668 that did not inhibit COX-2 expression. While VEGFR2 inhibitor treatment *prior to* VEGF activation almost completely abrogated expression of these genes, VEGFR2 inhibitor treatment *after* VEGF activation inhibited gene expression significantly less effectively. This demonstrates that endothelial cells that are engaged in angiogenic signal transduction exhibit an impaired reaction to blockade of VEGFR2 function. VEGFR2 inhibitor treatment of angiogenically activated HUVEC did not induce a change in phosphorylation of VEGFR2, p38 and Akt, but significantly inhibited ERK phosphorylation. The observation that this inhibition became effective only at >10 min after addition of vandetanib suggests that activated endothelial cells are refractory to VEGFR2 inhibition for a short period of time. Future studies will focus on analysis of other downstream effectors of VEGF signaling, such as Src, PLC γ and FAK, in response to pharmacological intervention with activated VEGFR2 signal transduction. Besides temporal effects, also shifts in signaling pathways employed under pharmacological pressure will be investigated.

INTRODUCTION

Tumors growing in an avascular niche require a neovascular bed to fulfill their continuous demand for oxygen and nutrients. This is accomplished through induction of angiogenesis, a hypoxia-driven, tightly regulated process in which vascular endothelial growth factor (VEGF) plays a key role. VEGF-A is one of the most potent angiogenic factors [1, 2], and expression of its receptor, VEGF-receptor 2 (VEGFR2), is upregulated in the vasculature of many tumors [3-5]. The notion that blockade of the blood supply inhibits tumor outgrowth has fuelled the development of numerous compounds that block angiogenesis, the majority of which target VEGF or its receptors [6].

VEGFR2 is the principle mediator of several physiological and pathological effects of VEGF-A on endothelial cells, while VEGFR1 is considered to play a main role as a decoy instead of a signaling receptor [7]. VEGFR2 activation promotes endothelial cell proliferation and migration, and induces vascular permeability [8, 9]. Furthermore, in endothelial cells VEGFR2 provides a survival signal through Akt-mediated deactivation of the pro-apoptotic proteins BAD and caspase 9, and induction of the anti-apoptotic proteins Bcl-2 and A1 [10-12]. Binding of VEGF leads to VEGFR2 dimerization, autophosphorylation of the receptor on a number of intracellular tyrosine residues, and subsequent activation of the intracellular signaling cascade. Phosphorylation of Tyr1175 allows binding and activation of phospholipase C γ , and subsequent generation of diacylglycerol and increased concentrations of intracellular calcium that activate protein kinase C (PKC). PKC activates the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase 1/2 (ERK1/2) cascade leading to proliferation of endothelial cells. Besides PLC γ , the adaptor molecule Shb binds to phosphorylated Tyr1175, resulting in activation of phosphoinositide 3-kinase (PI3K). PI3K activation regulates cell migration, but also endothelial cell survival through downstream activation of the serine/threonine kinase Akt/protein kinase B (PKB). Akt/PKB also regulates nitric oxide production by direct phosphorylation and activation of endothelial NO synthase (eNOS). Additionally, phosphorylation of Tyr1214 plays a role in vascular permeability through the subsequent activation of p38 MAPK and actin remodeling [10, 13].

Activation of endothelial cells *in vitro* with VEGF induces immediate upregulation of mRNA of the zinc finger transcription factor early growth response-3 (EGR3) and the NR4A family of orphan nuclear receptors [14, 15]. Both EGR3 and NR4A1 have been reported to function downstream of VEGF and mediate endothelial cell proliferation, migration and tube formation *in vitro*. *In vivo*, knock-down or inhibition of EGR3 and NR4A1 decreases microvascular density and growth of B16.F10 and B16.F1 melanomas

respectively [16, 17]. Additionally, VEGF stimulation rapidly induces mRNA expression of cyclooxygenase-2 (COX-2) [18], that is also known to stimulate tumor angiogenesis [19].

Several strategies have been designed to target VEGF signal transduction, including the use of small molecule receptor tyrosine kinase inhibitors. Most tyrosine kinase inhibitors prevent binding of ATP to the ATP-binding pocket of the VEGF receptor dimers, thereby preventing autophosphorylation. A number of clinical trials have demonstrated the benefit of these compounds in patients with *e.g.*, non small cell lung cancer and metastatic renal cell carcinoma [20-22]. Despite these important successes, many patients do not respond to VEGFR2 inhibitor therapy [23, 24].

The limited efficacy of antiangiogenic therapies may find its origin in heterogeneity of the molecular activation status of the tumor vasculature [25], in addition to the existence of evasive and intrinsic resistance [26]. It is highly likely that throughout the process of tumor growth, the endothelial cells are present in different angiogenic activation stages, in which the endothelial cells are engaged in temporally different steps of VEGFR2 signal transduction and downstream gene expression. We hypothesized that this difference in activation status renders endothelial cells differentially responsive to VEGFR2 inhibitor therapy. We therefore set out to investigate the consequences of angiogenic activation prior to treatment for responsiveness to VEGFR2 inhibitor therapy. For this we analyzed the ability of several VEGFR2 tyrosine kinase inhibitors (Table 1) to inhibit VEGF-induced immediate-early gene expression in endothelial cells that were activated with VEGF-A₁₆₅ (hereafter referred to as VEGF) prior to drug treatment. Such a situation, in which endothelial cells are treated with a VEGFR2 inhibitor after start of exposure to VEGF, is highly likely to be representative of the clinical setting, where (a subset of) tumor endothelial cells experience VEGF signaling even before VEGFR2 inhibitor therapy is initiated. To investigate the molecular mechanism underlying the impaired response, we analyzed the kinetics of phosphorylation of VEGFR2 and its downstream kinases ERK1/2, p38 MAPK, and Akt in response to VEGF stimulation, and inhibition thereof, both in an angiogenically activate and inactive endothelial cell state.

MATERIALS & METHODS

Endothelial cell culture and activation

Human umbilical vein endothelial cells (HUVEC) were obtained from the Endothelial Cell Facility of the University Medical Center Groningen, where they were

isolated from two umbilical cords to circumvent donor bias. Cells were cultured on 1% gelatin pre-coated culture flasks in RPMI 1640 (BioWhittaker/Lonza) supplemented with 20% fetal calf serum (FCS; Hyclone, Perbio Science, Etten-Leur, The Netherlands), 2 mM L-glutamin, 5 U/mL heparin (LEO Pharma BV, Breda, The Netherlands), 100 IE/mL penicillin (Astellas Pharma, Leiderdorp, The Netherlands), 100 µg/mL streptomycin (Rotex Medica, Trittau, Germany) and 50 µg/mL endothelial cell growth factor (ECGF) extracted from bovine brain [27] at 37°C in 5% CO₂/95% air in a humidified incubator. All experiments were performed with confluent monolayers cultured in 6-, 12-, or 96-wells plates with fresh HUVEC between passage 1 and 5.

Prior to each experiment, HUVEC were starved for 6h or overnight by culturing in medium that contained 2% bovine serum albumin (BSA; Sigma, Steinheim, Germany) instead of FCS and ECGF. HUVEC were stimulated with 5 ng/mL VEGF (recombinant human VEGF-A165, Biosource/Invitrogen, Camarillo, CA, USA) and treated with the VEGFR2 kinase inhibitors vandetanib (ZD6474; a kind gift from AstraZeneca, Macclesfield, UK), vatalanib (PTK787/ZK222584; Vichem Chemie Research Ltd, Budapest, Hungary), and SU6668 (a kind gift from Prof.Dr. G. Keri, Semmelweis University, Budapest, Hungary) at different concentrations and different time points relative to VEGF stimulation. For gene expression experiments, the cells were harvested for RNA extraction at 50 minutes after addition of VEGF, and for Western Blot the cells were harvested for protein analysis at 5, 15 and 30 minutes after VEGF stimulation.

Gene expression analysis by quantitative RT-PCR (qRT-PCR)

Total RNA was isolated using RNeasy Mini Plus kit (Qiagen, Leusden, The Netherlands), analyzed qualitatively by gel electrophoresis and quantitatively by Nanodrop ND-100 spectrophotometry (NanoDrop Technologies, Rockland, DE, USA), and reverse transcribed as described previously [28], using Superscript III Reverse Transcriptase (Invitrogen) in a 20 µL final volume containing 250 ng of random hexamers (Promega, Madison, WI, USA) and 40 units of RNase OUT inhibitor (Invitrogen). Real-time PCR was performed in duplicate per sample with 1 µL cDNA per reaction in TaqMan PCR MasterMix in a total volume of 10 µL, with primer-probe sets being purchased as Assay-on-Demand from Applied Biosystems (Nieuwekerk a/d IJssel, The Netherlands).

Western Blot detection of phosphorylated and total VEGFR2 and p38

HUVEC were treated as described above, washed with PBS and lysed in SDS sample buffer containing 62.5 mM Tris-HCL (pH 6.8), 2% SDS, 10% glycerol, 50 mM DTT, 1 mM sodiummorthovanadate (Sigma), and 0.01% bromophenolblue. Cells were scraped off

the plate and sonicated twice for 5 seconds to rupture DNA. After boiling for 5 minutes, the samples were separated onto a 7.5% (for detection of VEGFR2), 10% (for detection of Akt) or 12.5% (for detection of p38) SDS-PAGE gel, and blotted to a nitrocellulose membrane. The membrane was blocked in 5% dry milk in Tris-buffered saline containing 0.1% Tween-20 (TBS-T) and probed with Rabbit antibodies to phosphorylated VEGFR2 (Tyr1175), phosphorylated p38 MAPK (Thr180/Tyr182) or phosphorylated Akt (Ser 473; all three from Cell Signaling Technology, Danvers, MA, USA) diluted in 5% BSA/TBS-T overnight. The membranes were subsequently washed with TBS-T and incubated with horseradish peroxidase-labeled anti-rabbit IgG (Cell Signaling). After washing the membranes extensively in TBS-T, antibody binding was detected using chemi-luminescence (LumiLight Western Blotting Substrate; Roche Diagnostics, Mannheim, Germany).

Following detection of phosphorylated proteins, the membranes were stripped in stripping buffer (Thermo Scientific, Rockford, IL, USA) and reprobed with antibodies to the corresponding total (non-phosphorylated) VEGFR2, p38 and Akt (Cell Signaling) and antibody binding was detected as described above. Quantification of band intensity was performed using the Bio-Rad Quantity One 1D Gel Analysis software.

ELISA-based determination of phosphorylated and total ERK.

HUVEC were cultured in 96-wells plates and treated as described above. Phosphorylated and total ERK were detected using the Fast Activated Cell Based ELISA (FACE; Active Motif, Rixensart, Belgium), that was performed directly on the cultured cells (In-Cell) according to the supplier's protocol. Normalization to the number of cells per well was performed by staining with Crystal Violet (supplied in the kit) 1:3 diluted in PBS, followed by extensive washing in PBS and lysis of the cells with 1% SDS (supplied), after which absorbance was read at 595 nm.

Statistics

Statistical significance of the observed differences was addressed by means of ANOVA with post hoc comparison using Bonferroni correction. These statistical analyses were performed using GraphPad Prism version 5.00 (GraphPad Software, San Diego, CA, USA).

RESULTS

VEGF-induced immediate-early gene expression serves as a good read-out for VEGFR2 activity in HUVEC

We first established a method that could serve as a read-out for VEGFR2 signaling activity. Previous studies demonstrated that VEGF induces immediate mRNA upregulation of EGR3, NR4A1 and NR4A3 with a peak level at around 50 minutes after addition of VEGF, and also mRNA of COX-2 [14, 15, 18]. We activated HUVEC with VEGF at different concentrations (ranging from 1 to 50 ng/mL) and demonstrated that 5 ng/mL VEGF was sufficient to induce a >100-fold upregulation of EGR3 and NR4A1 mRNA levels, and a 7-15 fold upregulation of NR4A3 and COX-2 mRNA (data not shown).

We pre-treated HUVEC with different concentrations of the VEGFR2 tyrosine kinase inhibitors vandetanib (ZD6474), vatalanib (PTK787), and SU6668 (Table 1), followed by exposure to 5 ng/mL VEGF, and demonstrated that these inhibitors dose-dependently decreased VEGF-induced expression of EGR3, NR4A1 (Fig 1A-C) and NR4A3 (not shown). Vandetanib and vatalanib also decreased mRNA expression of COX-2, albeit to a lower extent as EGR3 and the NR4A nuclear receptors, while SU6668 did not affect mRNA levels of COX-2. As expected, the decrease in VEGF-induced gene expression upon vandetanib treatment was accompanied by a decrease in VEGFR2 phosphorylation levels (Fig 1D). Based on these data we concluded that, consistent with the prior reported IC_{50} values for the three drugs given in Table 1, 0.1-0.5 μ M of vandetanib and vatalanib was sufficient to effectively inhibit VEGF-induced gene expression, while for SU6668 a concentration of 2 μ M is necessary to prevent upregulation of these genes by VEGF. Using this setup we next investigated the kinetics of HUVEC responsiveness to VEGFR2 inhibitor therapy in varying experimental conditions.

Table 1. Molecular classification and activity of the drugs under study.

Drug	Drug class	Primary targets	IC_{50} (μ M)	References
Vandetanib (ZD6474; AstraZeneca)	Anilino derivative	VEGFR2 (KDR) [#]	0.04	[31]
		RET	0.13	[49]
		VEGFR3	0.11	
		EGFR	0.5	
Vatalanib (PTK787/ZK222584; Novartis)	Anilino derivative	VEGFR2 (KDR) [#]	0.037	[30]
		VEGFR1	0.077	
		PDGFR	0.58	
		c-Kit	0.73	
SU6668 (Sugen)	Indolinone	VEGFR2 (Flk1) [#]	2.1	[29,50]
		FGFR1	1.2	
		PDGFR	0.008	

[#]Affinity of vandetanib and vatalanib to VEGFR2 was tested in human cells (KDR), while that of SU6668 was tested in murine cells (Flk1)

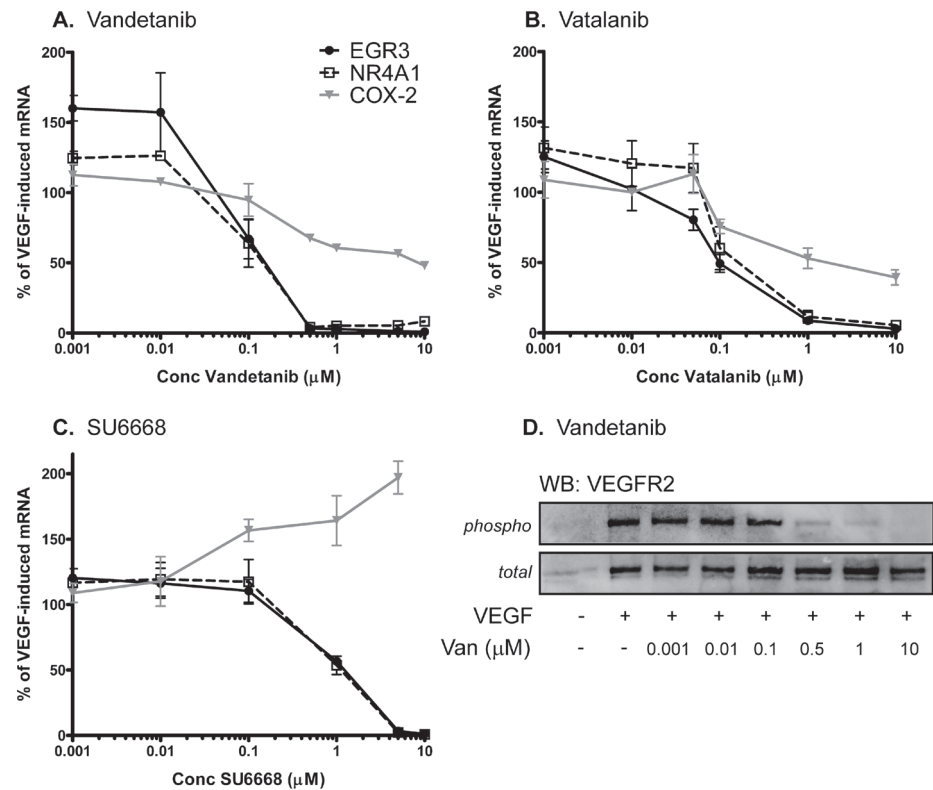


Figure 1: VEGFR2 tyrosine kinase inhibitor treatment dose-dependently inhibited VEGF-induced expression of immediate-early genes and VEGFR2 phosphorylation in HUVEC. A-C: HUVEC were treated with 5 ng/mL VEGF in combination with different concentrations of the VEGFR2 inhibitors vandetanib (A), vatalanib (B), and SU6668 (C), and gene expression of VEGF-induced immediate-early genes was analyzed at 50 minutes after VEGF stimulation. Values represent mRNA levels measured by qRT-PCR, adjusted to GAPDH, and expressed as percentage of VEGF-induced mRNA expression. Mean \pm SD, $n=2$ (for vandetanib) and $n=3$ (for vatalanib and SU6668). D: Western Blot detection of phosphorylated and total VEGFR2 in HUVEC treated with the indicated doses of vandetanib. Three independent experiments have been performed with similar results, one representative sample set is depicted.

Efficacy of VEGFR2 inhibitor therapy decreases when administered after angiogenic activation

We hypothesized that the activation status of endothelial cells at the moment of drug treatment influences their responsiveness to antiangiogenic therapy. We therefore investigated the consequences of treating HUVEC with VEGFR2 inhibitors *after* start of exposure to VEGF for the efficacy of VEGFR2 inhibitor activity. To mimic angiogenic

activation of endothelial cells, HUVEC were pre-treated with VEGF, and 5, 15 and 30 minutes later VEGFR2 inhibitors were added. While VEGFR2 inhibitor exposure prior to or simultaneously with VEGF activation almost completely abrogated expression of EGR3, NR4A1, NR4A3 and COX-2, addition of VEGFR2 inhibitors after start of exposure to VEGF was significantly less effective in inhibiting upregulation of these genes (Fig 2). When added 5 minutes after VEGF, vandetanib inhibited mRNA expression of EGR3

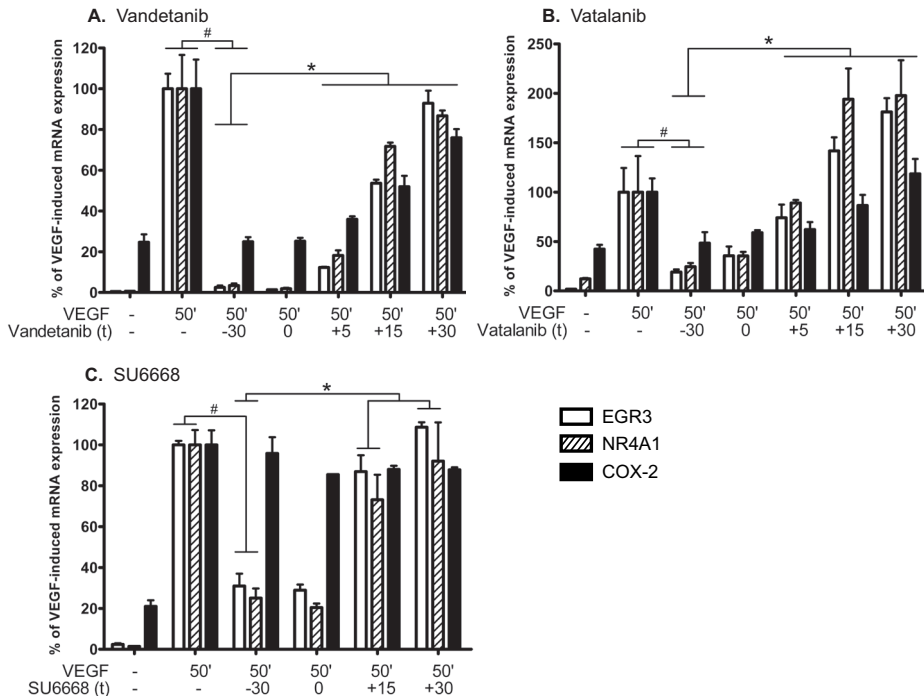


Figure 2: The inhibitory effect of vandetanib, vatalanib and SU6668 on VEGF-induced gene expression was reduced when the inhibitor was added after start of VEGF exposure. A-C: HUVEC were treated with 5 ng/mL VEGF in combination with the VEGFR2 inhibitors vandetanib (0.5 μ M), vatalanib (0.1 μ M) and SU6668 (2 μ M), that were added at different time points relative to start of VEGF exposure. Gene expression of VEGF-induced immediate-early genes was analyzed at 50 minutes after VEGF stimulation. Values represent mRNA levels measured by qRT-PCR, adjusted to GAPDH, and expressed as percentage of gene expression induced by VEGF alone. Mean \pm SD, $n=3$ (for vandetanib and vatalanib) and $n=2$ (for SU6668). Experiments have been performed 3-4 times with similar outcomes, one representative data set per drug is depicted. For clarity only a selection of statistical comparisons is indicated: # $p<0.001$ for VEGFR2 inhibitor treatment prior to VEGF compared to VEGF stimulation alone, and * $p<0.001$ for VEGFR2 inhibitor treatment after VEGF compared to treatment prior to VEGF exposure. Gene expression levels upon exposure to VEGF alone were statistically tested against the levels in untreated control and found significant ($p<0.001$), yet for clarity of the figure this is not depicted.

and NR4A with 80-85%, yet when the inhibitor was added 30 minutes after VEGF, the inhibitory effect declined to 8-10%. Three independent experiments with HUVEC from different donors showed similar outcomes (data not shown). Analogous results for EGR3, NR4A1 and NR4A3 responsiveness were observed upon treatment with vatalanib and SU6668 (Fig 2 and data not shown). Late treatment with vatalanib even upregulated EGR3 and NR4A1 mRNA 2-fold compared to the mRNA levels induced by VEGF alone in two out of three experiments. Altogether, these data demonstrate that the efficacy of VEGFR2 inhibitor therapy was strongly diminished when administered to angiogenically active endothelial cells compared to quiescent endothelial cells. Furthermore, the observation that SU6668 did not inhibit COX-2 expression, in contrast to vandetanib and vatalanib, suggests that different VEGFR2 inhibitors differentially affect downstream kinases and transcription factors.

Angiogenic activation-related differences in the molecular effects of VEGFR2 inhibition on downstream kinase phosphorylation

Our data suggest that, once activated by VEGF, endothelial cells are less sensitive to VEGFR2 inhibitor treatment, as exemplified in regulation of gene expression. Since kinases represent the upstream driving force of transcription factor activation and gene expression, we investigated the level of phosphorylation of VEGFR2 and of its downstream kinases p38 mitogen-activated protein kinase (p38 MAPK), Akt and extracellular signal-regulated kinase-1/2 (ERK1/2, hereafter referred to as ERK). We studied the kinetics of activation of these pathways under VEGF pressure alone as well as in response to VEGFR2 inhibitor treatment, both in a pre-treatment (quiescent cell status) setting in which drugs were added prior to VEGF, and in an angiogenically active cell status where drugs were added after exposure to VEGF. As an initial step we chose vandetanib, but given the differential activation of VEGF-responsive genes, future research will study the effects of SU6668 and vatalanib on kinetics of VEGFR2 signal transduction as well.

In response to VEGF, VEGFR2 phosphorylation was significantly induced, and remained phosphorylated until 30 min after VEGF stimulation (Fig 3A). This resulted in downstream activation of ERK, which had a peak level at 5-15 min and declined to ~60% of its maximum level after 30 min, still representing a significant level of phosphorylation compared to that of the untreated control (Fig 4). In contrast, p38 and Akt phosphorylation levels did not significantly change upon VEGF stimulation (Fig 3B+C). Of note is the observation that high constitutive levels of phosphorylation of p38 and Akt were observed even in culture conditions without serum, whereas these conditions almost completely

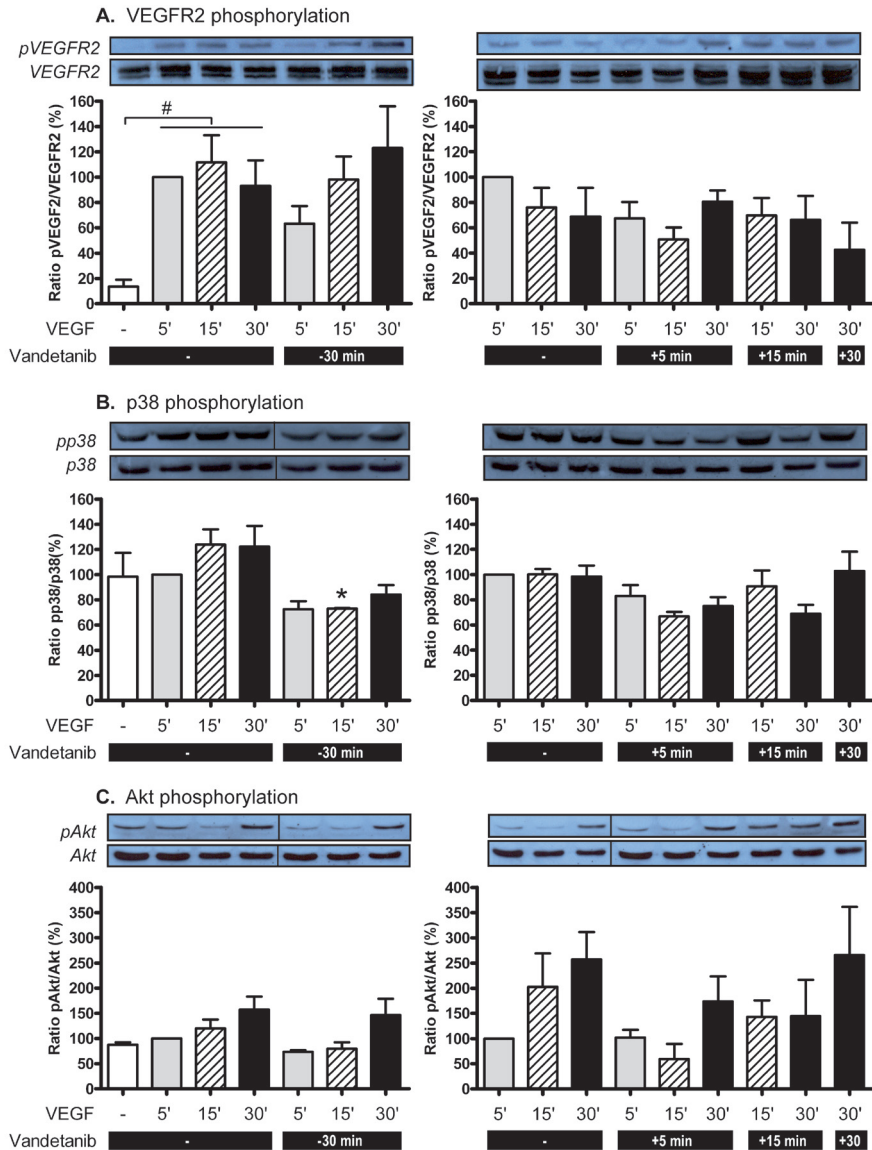


Figure 3: Kinetics of VEGFR2 and downstream p38 and Akt phosphorylation upon treatment with VEGF and vandetanib at different time points. A-C: HUVEC were treated with 5 ng/mL VEGF and 1 μ M vandetanib was added at different time points relative to VEGF stimulation. At 5, 15 and 30 minutes after start of VEGF exposure, cells were harvested and the levels of phosphorylated and total VEGFR2 respectively p38 and Akt were determined by Western Blot. Values represent quantification of band intensity, expressed as percentage of VEGF-induced phosphorylation after 5 minutes. Mean + SEM of 4 (VEGFR2, Akt) or 3 (p38) independent experiments. Significance of only a selection of comparisons is indicated: [#] $p < 0.05$ compared to untreated control, and ^{*} $p < 0.05$ for VEGFR2 inhibitor treatment compared to treatment with VEGF alone at the corresponding time points.

abolished VEGFR2 and ERK phosphorylation. Possibly, this basal level of p38 MAPK and Akt phosphorylation did not allow an increase in phosphorylation under VEGF pressure.

Pre-treatment with vandetanib prior to VEGF exposure induced an initial, yet statistically not significant reduction of VEGFR2 phosphorylation at 5 minutes after start of VEGF exposure, after which the inhibitory effect disappeared. In addition, vandetanib pre-treatment significantly inhibited p38 activation at 15 minutes after start of VEGF exposure when compared to untreated, VEGF-exposed control, but did not affect Akt phosphorylation. ERK phosphorylation was inhibited by pre-treatment with vandetanib throughout the 30 minutes time period. This suggests that while the inhibitor gradually lost its capacity to inhibit VEGFR2 phosphorylation, downstream ERK kinase activation remained inhibited throughout at least 30 minutes.

In angiogenically activated HUVEC, vandetanib treatment did not induce any significant changes in the phosphorylation level of VEGFR2, p38 and Akt. At early time points, it also could not instigate inhibition of ERK phosphorylation. However, 10 minutes after addition of vandetanib, we observed significant inhibition of ERK phosphorylation which lasted until 25 minutes after treatment.

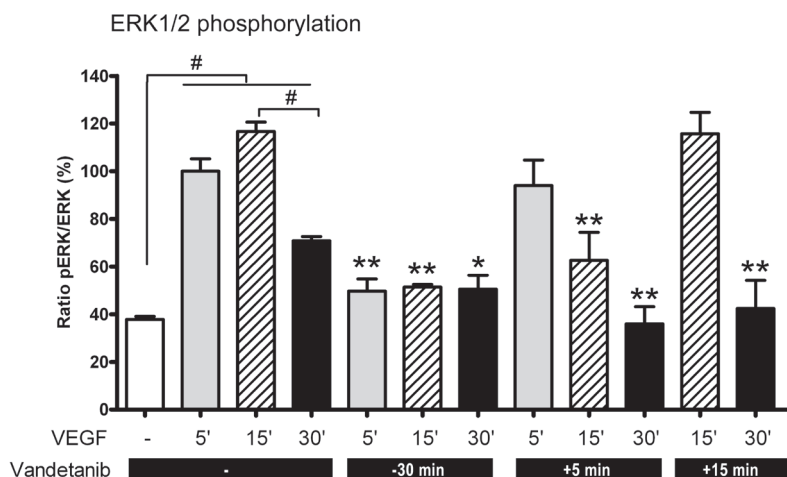


Figure 4: Kinetics of ERK phosphorylation upon treatment with VEGF and vandetanib at different time points. Detection of phosphorylated and total ERK by In-cell ELISA in HUVEC treated with 5 ng/mL VEGF and 1 μ M vandetanib at the indicated time points. Mean + SD, n = 3. Three independent experiments with HUVEC from different donors gave similar results, one representative sample set is depicted. For clarity of the figure, significance of only a selection of comparisons is indicated: #p<0.05 for the indicated comparisons, and *p<0.05 and **p<0.0001 for VEGFR2 inhibitor treatment compared to treatment with VEGF alone at the corresponding time points.

DISCUSSION

Thus far, the majority of *in vitro* studies that evaluate VEGFR2 inhibitors for their capacity to inhibit VEGFR2 signaling and function have been performed in serum-starved quiescent endothelial cells [29-34]. In the complex *in vivo* tumor environment, however, endothelial cells experience continuous, though dynamically changing levels of VEGF. A certain percentage of tumor endothelial cells likely exist in an angiogenically activated state at the moment treatment with VEGFR2 inhibitors is initiated. We hypothesized that a pre-existent angiogenic active status of the endothelial cell would influence its responsiveness to pharmacological inhibition of VEGFR2. Therefore, we studied *in vitro* whether angiogenic activation of endothelial cells prior to start of treatment influences the efficacy of VEGFR2 inhibitor treatment. We evaluated this by studying the capacity of the drug to inhibit expression of VEGF-responsive genes. Our data demonstrate that VEGFR2 inhibitor treatment administered *after* angiogenic activation of the endothelial cells has an impaired ability to block VEGFR2 signaling. Analysis of the underlying kinetics of phosphorylation of VEGFR2, ERK, Akt and p38 revealed that, once VEGF signaling is activated, ERK showed a delay in response to VEGFR2 inhibitor treatment. At the same time, no significant changes in the phosphorylation levels of VEGFR2, p38 and Akt were detected. Our data hence suggest that endothelial cells engaged in VEGF signaling remain refractory for VEGFR2 inhibitor treatment for a short period of several minutes. Further studies will focus on assessing the phosphorylation kinetics of these and other kinases involved in VEGF signal transduction in response to VEGFR2 inhibitor treatment, to broaden our understanding of the time frame in which VEGFR2 inhibitor treatment affects the various pathways downstream of VEGF.

We demonstrated that VEGF induced significant phosphorylation of the VEGFR2 tyrosine residue 1175 in HUVEC, achieving a peak level of phosphorylation at 5-10 minutes and remaining in a phosphorylated state until 30 minutes after start of exposure. Several other studies reported faster VEGFR2 phosphorylation kinetics that peaked at 3-5 minutes after ligand stimulation and declined by 10-15 minutes [35-37]. These studies analyzed phosphorylation at a different tyrosine residue (Tyr1054/1059), or were carried out in a different type of endothelial cells (porcine aortic endothelial cells) and with a higher concentration of VEGF, which may explain the observed different kinetics of Tyr1175 phosphorylation.

Addition of vandetanib to endothelial cells in quiescent state initially prevented VEGFR2 phosphorylation, thereby preventing ERK and p38 phosphorylation as well. Yet, while VEGFR2 phosphorylation levels gradually increased even under

4 vandetanib-pressure, p38 and ERK phosphorylation levels remained inhibited for the full 30 min, consistent with the observation that expression of VEGF-responder genes was almost completely abrogated throughout the 50 minutes period studied. A pharmacological interesting observation was that treatment of angiogenically active HUVEC with vandetanib did not inhibit VEGFR2 phosphorylation, but significantly inhibited ERK phosphorylation at >10 min after vandetanib administration. This suggests that vandetanib did not induce detectable changes at the surface receptor level, while remarkably affecting downstream ERK. Data in a human head and neck carcinoma cell line suggest that vandetanib can directly inhibit ERK activation, as the authors observed decreased ERK phosphorylation levels upon vandetanib treatment even in the absence of the target receptor EGFR [38]. Yet these cells secrete VEGF in normal conditions, and the possibility that vandetanib inhibited ERK phosphorylation through inhibition of VEGFR2 activity in this set up has not been addressed. As for the difference between VEGFR2 and ERK responses to vandetanib, it should be taken into account that In-Cell ELISA-based detection of phospho-ERK may be considered more powerful to quantitatively detect changes in the phosphorylation level than Western Blot detection of phospho-VEGFR2.

In contrast to vandetanib and vatalanib, SU6668 did not inhibit COX-2 expression in HUVEC, while effectively preventing EGR3, NR4A1 and NR4A3 upregulation upon VEGF exposure. The induction of EGR3 and NR4A1 mRNA is mediated predominantly by PKC-ERK1/2 signaling [14], while COX-2 expression requires recruitment and activation of p38 MAPK, but not of ERK [18]. This suggests that SU6668 does not inhibit p38 phosphorylation, while vandetanib and vatalanib do. For SU6668, additional studies will be performed to investigate whether the lack of COX-2 inhibition can be attributed to lack of p38 MAPK inhibitory activity. The high levels of p38 in the absence of VEGF may explain the relative insensitivity of p38 phosphorylation to VEGFR2 inhibitor treatment, and perhaps also the relative small induction of COX-2 expression, as compared to EGR3 and NR4A, in response to VEGF stimulation.

Recent studies have demonstrated that resting endothelial cells have two surface pools of VEGFR2, a stable pool that is complexed with VE-cadherin, thereby preventing VEGFR2 from internalization, and a fluxing pool that is constantly cycling between the cell membrane and the sorting endosomes [39-41]. In unstimulated conditions, approximately 40% of total VEGFR2 resides in an intracellular compartment [42]. Stimulation with VEGF leads to internalization of the receptor and part of this is next targeted for degradation through the proteasome pathway or in the lysosomes [13, 43], while another part is shuttled back to the cell surface. In fact, VEGF stimulation has

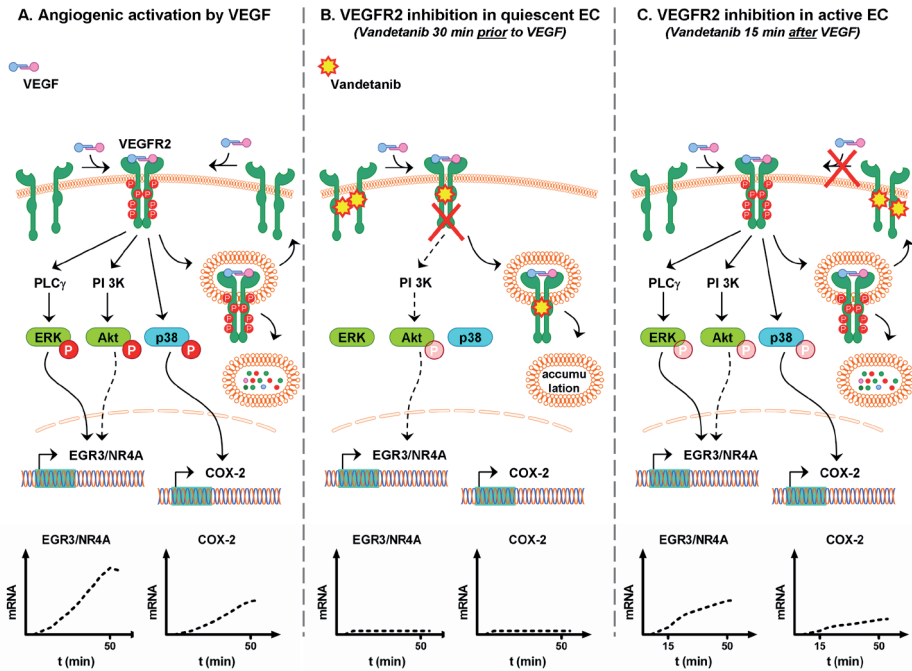


Figure 5: Hypothetical scheme summarizing our findings on the impaired responsiveness of angiogenically activated endothelial cells to VEGFR2 inhibitor therapy. **A:** Upon ligand binding, VEGFR2 induced immediate-early expression of EGR3, NR4A1 and NR4A3 mRNA mediated by ERK, while NR4A3 is also slightly induced by Akt. VEGF induced expression of COX-2 via recruitment and activation of p38 MAPK [14, 18]. **B:** Pre-treatment of HUVEC with vandetanib and vatalanib prior to VEGF stimulation almost completely blocked the induction of gene expression for 50 minutes, which was accompanied by a significant inhibition of p38 and ERK for at least 30 minutes, but not Akt. **C:** Inhibition of VEGFR2 at 15 minutes after angiogenic activation of endothelial cells resulted in 30-50% inhibition of VEGF-induced EGR3/NR4A expression and 50% inhibition of COX-2 expression at 50 minutes. We could not detect any significant changes in VEGFR2, p38 and Akt phosphorylation level immediately after or 15 minutes after vandetanib addition, yet we demonstrated that ERK phosphorylation was decreased 15 minutes after vandetanib was added. This delay in inhibition of ERK phosphorylation may involve VEGF binding-induced redistribution of VEGFR2 within the cell. While a new pool of VEGFR2 is trafficking to the cell surface, the cell is rendered refractory for VEGFR2 inhibition for a short time. We could not detect a significant change in p38 phosphorylation, but the decrease in COX-2 expression suggests that also p38 phosphorylation is inhibited by vandetanib. Our data suggest that Akt is not inhibited by VEGFR2 inhibitor treatment. Note that this model does not take into account the kinetics of responder gene mRNA production and degradation. Furthermore, while some signaling pathways do not depend on endocytosis and can occur starting from the plasma membrane, MAPK signaling requires receptor endocytosis [39], however for clarity of the figure we provide here a simplified scheme.

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been reported to mobilize the intracellular store of VEGFR2 and provoke the return of internalized receptor through an endocytic recycling pathway [42]. Notably, treatment of endothelial cells with the VEGFR2 inhibitor SU5416 blocked VEGFR2 phosphorylation and ligand-induced degradation of the receptor, but did not affect binding of VEGF and internalization, leading to endosomal accumulation [35]. This suggests that when endothelial cells are exposed to the VEGFR2 inhibitor prior to or simultaneously with VEGF stimulation, endosomal accumulation of VEGFR2 upon VEGF binding renders the receptors refractory to ligand stimulation. This explains the effective inhibition of gene expression throughout the full 50 minutes. In angiogenic conditions, the recycling process of ligand-bound VEGFR2 may account for the lag time between drug addition and inhibition of ERK phosphorylation. Our findings on the kinetics of VEGFR2 inhibitor effects are summarized in Figure 5, in a schematic model describing the molecular events that mediate the response of quiescent and angiogenically active endothelial cells to therapy. This model does not take into account the kinetics of VEGFR2 trafficking, and the observation that VEGFR2 residing in an intracellular compartment maintains its signaling capacity, nor that receptor endocytosis is required for kinase activation, as is the case for MAPK signaling [39, 41]. Other factors that regulate VEGFR2 activity, such as dephosphorylation by phosphatases like vascular endothelial protein tyrosine phosphatase (VE-PTP) or Src-homology phosphatase-1 and 2, are not taken into account in this schematic model either [44, 45]. Furthermore, the presence of heparan sulphate proteoglycan (HSPGs), complex formation with integrin- β 1 and binding of VEGFR2 to its co-receptor neuropilin are known to determine the level of VEGFR2 phosphorylation, and activation kinetics of downstream p38 MAPK [36, 46]. These issues all need to be considered to obtain a better picture of the molecular response of endothelial cells to pharmacological inhibition of VEGFR2.

Detailed knowledge of the molecular pharmacology of VEGFR2 inhibition is still lacking, and many questions remain unanswered. If the receptor is in a ligand-bound condition, does it remain refractory for drug treatment? And once the drug is bound to the receptor, how long does it prevent autophosphorylation of the receptor? Are the effects dependent on VEGFR2 density on the membrane and the expression levels of VE-cadherin? How long do downstream kinases other than ERK, p38 and Akt remain in a phosphorylated state when upstream VEGFR2 activation is blocked? And once the VEGFR2-activated kinases are inhibited, how long do they remain in an inactive state? The kinetics of these events remain elusive. A detailed analysis of VEGFR2 and downstream phosphorylation kinetics upon VEGFR2 inhibitor treatment requires the availability of highly sensitive techniques for quantification of phosphorylation levels of a

variety of kinases involved in the signaling pathway. Furthermore, investigating whether the observed delay in inhibition of VEGF signaling involves intracellular VEGFR2 trafficking, redistribution and interactions with signaling or adaptor molecules, requires techniques such as Fluorescence Resonance Energy Transfer (FRET) in combination with confocal microscopy life cell imaging protocols that enable real-time visualization of the VEGFR pathway. For many of these studies, endothelial cell lines for efficient knock-in and knock-out modifications are an essential asset, yet molecular control of their behavior may be even further off the *in vivo* behavior than that of primary endothelial cells.

In conclusion, our study demonstrated that the pharmacological efficacy of VEGFR2 inhibitors is impaired when administered to endothelial cells that are already engaging in VEGF-activated angiogenic signaling. Analysis of phosphorylation levels suggested that treatment of angiogenic active endothelial cells did not significantly affect VEGFR2 phosphorylation, yet ERK phosphorylation was inhibited with a delay of approximately 10 minutes. Further studies will focus on quantification of phosphorylation of other kinases involved in the VEGF signaling transduction, such as Src, PLC γ , PI3K and FAK, to dissect the consequences of VEGFR2 inhibitor treatment on activation of these pathways. These kinases could be analyzed using In-Cell ELISA, signal transduction arrays [36] or by kinome-wide profiling [47]. Finally, extension of the time periods studied can reveal whether prolongation of VEGFR2 inhibitor pressure can overcome the impaired efficacy. Validation of these findings in *in vivo* tumor models is essential, as cells that are taken out of their microenvironmental context lose their *in vivo* phenotype and molecular behavior [48].

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